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# DIFFERENTIAL SENSITIVITY OF RETICULOCYTES TO NICKED AND UNNICKED DIPHTHERIA TOXIN

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## SUMMARY

The effects of diphtheria toxin (DT) on rabbit reticulocytes were studied. Protein synthesis of the cells was inhibited at relatively high DT concentrations. Several drugs including metabolic inhibitors, nucleotides and amines blocked DT-induced toxicity in reticulocytes in a manner comparable to their known protective effects in other cell lines. This suggested that receptor-mediated binding and internalization of DT occurred in reticulocytes. Highly purified unnicked DT did not inhibit protein synthesis, while nicked toxin was effective. Treatment of the unnicked toxin with trypsin converted it into a potent form. It is hypothesized that reticulocytes lack the required toxin-activating (nicking) enzyme or that the putative enzyme is not located on or in the cell in a manner where it can interact with the toxin.

Diphtheria toxin (DT) is a potent cytotoxin for many eukaryotic cell lines (for a review, see [1]). As elaborated by the organism, DT is a single polypeptide of 60 000 D. Depending on the preparation, a variable fraction of the toxin is cleaved in the polypeptide backbone (the "nicked" form). This cleavage produces two fragments which are held together by an interfragment disulfide and non-covalent forces. The smaller of the two fragments, fragment A, catalyzes the transfer of the adenosine diphosphoribose portion of nicotinamide adenine dinucleotide to a protein acceptor (see below). The larger of the two fragments, fragment B, is required for receptor recognition and binding of the toxin. Thus, while some important details are lacking, the general features of the toxin's mechanism of action are as follows: the toxin initially binds to a specific receptor found on the surface of susceptible cells. By an ill-defined process, fragment A enters the cell, ultimately reaching the cytoplasmic compartment where it inactivates elongation factor 2, a eukaryotic enzyme essential for protein synthesis. The intoxicated cell then dies due to its inability to synthesize proteins.

One of the most intriguing questions about DT's mechanism of action is how the toxin (or fragment A) reaches the cytoplasm in an intact, functional form. One suggestion is that DT directly traverses the plasma membrane after binding to its receptor [2]. Another possibility [3] is entry by receptor-mediated endocytosis (RME), a route followed by several macromolecules, hormones and viruses. Basically, RME involves ligand binding to a receptor and subsequent uptake into vesicles which may fuse with lysosomes. It is hypothesized that a fraction of the toxin somehow escapes lysosomal degradation and enters the cytoplasm [4].

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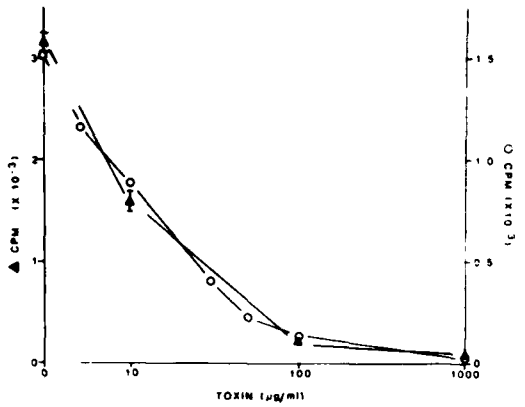


*Fig. 1.* Effect of DT on protein synthesis of reticulocytes. Freshly isolated cells (▲) or cells isolated and held overnight at 4°C (○) were incubated with the indicated concentration of DT for 3 h at 37°C and protein synthesis measured as in Materials and Methods (triplicates).

The hallmark of RME is delivery to and processing by the lysosome. Therefore, it would be interesting to study the effects of DT on cells that lack this organelle. It is believed that the maturing reticulocyte is markedly, if not totally, depleted of lysosomes. We therefore studied the toxicity of DT for reticulocytes and report the findings here. Protein synthesis in isolated reticulocytes is inhibited by DT and the response has many features of a receptor-mediated process. Most interesting was the observation that unnicked DT is non-toxic to these cells.

## MATERIALS AND METHODS

### *Cells*

Reticulocytes were obtained from male New Zealand white rabbits. The animals were given 8 mg/kg phenylhydrazine on days 1–3, and 4 mg/kg on day 4. On days 7–9, 2–3 ml blood was drawn into heparinized syringes and the reticulocytes isolated and washed by centrifugation at 600 g for 5 min, using Hanks' Balanced Salt Solution (HBSS). Untreated animals had hematocrits of 35–40%, while hematocrits of phenylhydrazine-treated animals ranged from 10 to 20% with a median about 12. The final preparations were about 80–90% reticulocytes as determined by light microscopy.

Table 1. *Effect of metabolic inhibitors on diphtheria toxin-induced protein synthesis inhibition*[a]

| Drug | Conc. (mM) | Protein synthesis (cpm) Drug alone | Drug and toxin |
|---|---|---|---|
| None | | 2 200 | 400 (18%)[b] |
| Fluoride | 0.5 | 2 150 | 530 (24%) |
| | 1.0 | 1 970 | 780 (40%) |
| | 3.0 | 1 400 | 1 300 (93%) |
| Azide | 0.5 | 1 870 | 670 (38%) |
| | 1.0 | 2 040 | 1 030 (51%) |
| Cyanide | 1.0 | 1 940 | 550 (28%) |
| | 5.0 | 1 500 | 430 (29%) |
| Dinitrophenol | 0.05 | 2 000 | 1 000 (50%) |
| | 0.1 | 1 380 | 1 380 (100%) |
| | 0.5 | 1 600 | 1 680 (105%) |
| Deoxyglucose | 30 | 1 980 | 330 (13%) |
| | 100 | 1 710 | 350 (20%) |
| Salicylate | 3.0 | 2 650 | 670 (25%) |
| | 10 | 2 120 | 1 230 (58%) |

[a] Cells were incubated with the indicated agent for 1 h at 37°C. Toxin was added (100 μg/ml) and incubation continued for 2.5 h. Toxin and drug were washed out and the cells were incubated 1 h further at 37°C. Protein synthesis was then measured with a 1 h pulse of [$^3$H]leucine as outlined in Materials and Methods.
[b] % of drug control.

### *Toxicity assay*

Cells were suspended in Hanks' 199 medium with 10% fetal calf serum (FCS) and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) at concentrations of $\approx 3\times10^7$/ml. Individual assays were performed with 0.2 ml of cell suspension. Drugs and toxin were added and incubations carried out at the temperature and for the times indicated. Protein synthesis was assessed with a 1 h pulse of 1 μCi/tube of [$^3$H]-leucine (New England Nuclear). The pulse was terminated by centrifugation, removal of the medium, and addition of 0.5 ml of 0.15% sodium dodecylsulfate (SDS). The entire sample was transferred onto a numbered 11 mm paper disc (Schleicher and Schuell, no. 740E) and placed in a bottle of 10% trichloroacetic acid (TCA). The discs were washed twice with 5% TCA, twice with 1:1 ethanol:ether, and once with ether. After drying, the discs were counted in a toluene-based liquid scintillation solution (Liquifluor, New England Nuclear).

### *Toxin*

Diphtheria toxin (Lot nos. D-298 and 379) was purchased from Connaught Laboratories (Toronto) and purified by chromatography over DE-52. At that stage Lot no. D-298 was ≥99% pure and ≈90% nicked, while Lot no. 379 was also ≥99% pure but

95% unnicked, as judged by SDS polyacrylamide gel electrophoresis (SDS–PAGE). To obtain "totally" unnicked toxin, purified Lot no. 379 material was incubated with 0.5 M guanidine hydrochloride and dithiothreitol for 30 min at 25°C, then dialyzed into HBSS. This treatment served to separate the A and B fragments of the nicked contaminating toxin which, under our experimental conditions, apparently did not reassociate. Using radiolabeled toxin and SDS–PAGE, we judged this final toxin preparation to be ≥99% unnicked.

## RESULTS

A dose-response curve for inhibition of reticulocyte protein synthesis by nicked DT is shown in fig. 1. Compared to other cells from susceptible animals [5], quite high concentrations of DT are required to obtain inhibition. The triangles show the response of cells obtained on the same day as the challenge with toxin. The circles represent the same cells held overnight at 4°C and then challenged the next day with toxin. Whereas the total incorporation of radiolabel was reduced about 50% in cells held overnight, it is obvious that a similar dose-response was obtained. This observation was technically useful, in that cells prepared on one day could be used the next. The assay was reproducible, the bars showing standard errors which were, in most cases, smaller than the size of the symbols. Red cells prepared in exactly the same manner from animals not treated with phenylhydrazine incorporated only 2–4% of the radiolabel incorporated by reticulocytes. This indicates that neither adult red cells nor contaminating white cells would contribute significantly to our assay (white cell numbers (≈0.1%) were similar in both preparations).

In other systems where DT toxicity is believed to be receptor-mediated [6–8], a number of drugs or chemical agents have been shown to protect from the toxin. We investigated the effects of several classes of DT-protective agents in the reticulocyte system. Table 1 shows the results of experiments with metabolic inhibitors. In general, those metabolic inhibitors previously reported to be DT-protective were also protective for reticulocytes. Fluoride, azide, and salicylate all inhibited the action of DT as had been observed with Vero [9] and other cell lines [7]. Cyanide and deoxyglucose, agents which provided little or no protection to other cells, were likewise ineffective with reticulocytes. A notable exception to these correlations was dinitrophenol. This drug, previously found to provide essentially no protection from DT [7–9], was the most potent protective metabolic inhibitor tested in the reticulocyte system.

A second class of compounds noted for their DT-protective effects are nucleotides [10]. Table 2 shows the pattern of protection exhibited by these agents in the reticulocyte system. As previously observed

Table 2. *Protection of reticulocytes from diphtheria toxin by nucleotides*[a]

| Nucleotide | Conc. (mM) | Protein synthesis (cpm) Nucleotide alone | Nucleotide + toxin |
|---|---|---|---|
| None | | 2 120 | 930 |
| Adenosine triphosphate | 1 | 2 340 | 1 320 |
| | 3 | 1 980 | 1 220 |
| Adenosine tetraphosphate | 1 | 2 550 | 1 610 |
| | 3 | 1 720 | 1 730 |
| Cytosine triphosphate | 1 | 2 300 | 1 010 |
| | 1 | 2 220 | 1 130 |
| Guanosine triphosphate | 1 | 2 490 | 1 520 |
| | 3 | 2 070 | 1 660 |
| Thymidine triphosphate | 1 | 1 980 | 1 280 |
| | 3 | 1 970 | 1 590 |
| Thymidine tetraphosphate | 1 | 2 375 | 2 140 |
| | 3 | 2 070 | 1 760 |
| Tripoly-phosphate | 1 | 1 990 | 950 |
| | 3 | 1 480 | 1 040 |
| Control no. 2 | | 2 070 | 950 |

[a] Nucleotides, then toxin (50 μg/ml) were added to the cells and incubation carried out for 3 h at 37°C. Protein synthesis was then measured with a 1 h pulse of [$^3$H]leucine as outlined in Materials and Methods.

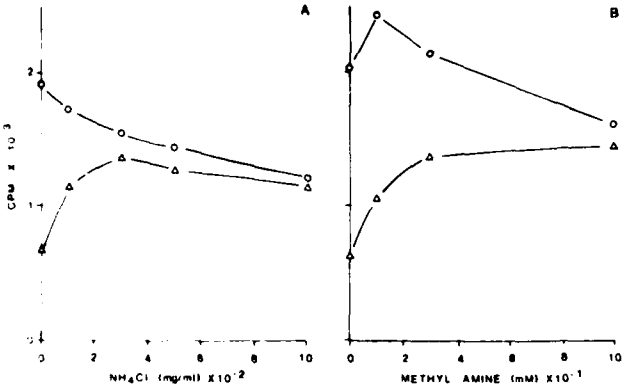


*Fig. 2.* Protection of reticulocytes from DT by ammonium chloride and methyl amine. Cells were incubated for 30 min at 37°C with the indicated concentration of drug. Toxin (50 μg/ml final) was added to one-half of the cells and incubation continued for 3 h at 37°C. Protein synthesis was then measured in (△) toxin or (○) non-toxin-treated cells as in Materials and Methods.

with Vero cells [10], the tetraphosphate nucleotides were more potent than the triphosphates. Also as with Vero cells, we found that thymidine, adenosine, and guanosine triphosphates exhibited some protection, whereas cytosine triphosphate and tripolyphosphate did not.

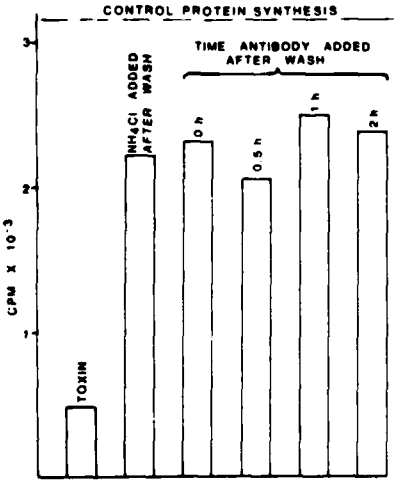


*Fig. 3.* Lack of requirement for antitoxin with ammonium chloride protection from DT. Cells were incubated with $NH_4Cl$ (0.08 mg/ml) for 30 min at 37°C and then with 100 μg/ml toxin for 3 h at 37°C. Cells were rinsed 2× and incubation continued for an additional 2 h with medium alone (bar labeled toxin), medium containing 0.08 mg/ml ammonium chloride (bar labeled $NH_4Cl$ added after wash), or medium to which antitoxin was added at the specified times. At the end of the 2 h period, protein synthesis was measured with a 1 h pulse using [$^3$H]leucine.

Another class of agents with interesting DT-protective properties are the simple organic amines [4, 7, 11–13]. Fig. 2 shows that both ammonium chloride and methylamine protect reticulocytes from DT. By themselves, both these agents had modest but variable inhibitory effects on protein synthesis; nevertheless, drug protection was always observed. Chloroquine, a more widely employed lysosomotropic amine, also protected reticulocytes from DT (data not shown).

An interesting feature of ammonium chloride protection from DT in other cell systems is the requirement for antitoxin treatment upon removal of the drug. Thus, Kim & Groman [11] and Ivins et al. [7] found that cells incubated with DT and ammonium chloride did not survive when the drug was washed out. However, if antitoxin were added immediately following the wash, protection was complete. Using similar protocols, a requirement for antitoxin was not observed with reticulocytes. Fig. 3 shows that preincubation of cells with toxin followed by washing and a further 2-h incubation resulted in substantial inhibition of protein synthesis (first bar). If ammonium chloride was present both during the preincubation with toxin and during the 2 h period after toxin washout, considerable,

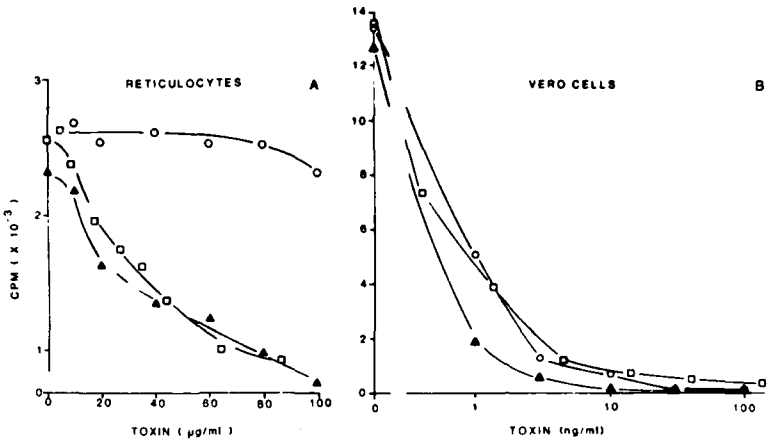


*Fig. 4.* Effect of nicked and unnicked DT on protein synthesis in reticulocytes and Vero cells. Cells were incubated for 3 h with the indicated concentration of DT and protein synthesis measured as in Materials and Methods. ○, Guanidine-DTT-treated "unnicked" toxin; □, "unnicked" toxin (≃4 mg/ml) treated with 1 μg/ml trypsin for 30 min at 25°C; ▲, prenicked toxin.

but not complete protection was observed (second bar). Interestingly, if ammonium chloride was present only during the toxin preincubation period, a comparable level of protection was observed (2 h bar). Moreover, the addition of antitoxin immediately or at any timepoint after toxin and ammonium chloride washout did not confer additional protection. Thus, whereas ammonium chloride protects reticulocytes from DT, it is clear that some features of the protection differ from those operative in other DT-sensitive cells.

The DT preparation used for experiments shown thus far was substantially (≃90%) nicked. We obtained a separate toxin lot from Connaught Laboratories that was primarily unnicked (95%). After purification in the usual manner (see Materials and Methods), the toxin was tested in the reticulocyte system and found to have much lower specific activity than the nicked preparation (data not shown). To remove the small fraction of nicked toxin, the purified preparation was treated further with guanidine hydrochloride and DTT to obtain a preparation that was ≤1% nicked. The guanidine-DTT-treated toxin was virtually non-toxic to reticulocytes (fig. 4*A*). Incubation with trypsin resulted in a substantial activation to a specific activity indistinguishable from the toxin lot that was ≃90% "naturally" nicked. There were only small and non-reproducible differences in the toxicity of nicked or unnicked DT using Vero cells (fig. 4*B*).

In order to develop a further correlation between nicking and activation of toxicity for reticulocytes, DT was treated with trypsin for various time lengths and then tested for toxicity. Fig. 5 shows that the longer the unnicked DT was incubated with trypsin, the more toxicity it exhibited. SDS–

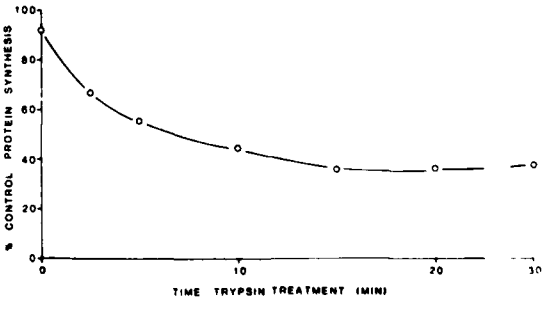


*Fig. 5.* Activation of unnicked DT by trypsin treatment. Guanidine-DTT-treated toxin (≃4 mg/ml) was incubated with 1 μg/ml trypsin for the time specified. The reaction was stopped by addition of soybean trypsin inhibitor to 3 μg/ml. Toxicity was tested by a 3 h incubation with 50 μg/ml of DT.

PAGE gels showed a progressive nicking with time, starting at not detectable and ending (30 min) at 70–75%.

## DISCUSSION

It is clear from the experiments reported here that diphtheria toxin can inhibit protein synthesis in rabbit reticulocytes. The high concentrations of toxin required for inhibition could reflect a low efficiency of receptor-mediated toxin delivery to the cytoplasm due to deficiencies at any one or more steps (reduced receptor numbers or binding affinity, reduced transport rates, etc.). Alternatively, toxicity could be due to non-specific uptake processes. Attempts to measure toxin-reticulocyte binding by methods similar to the Vero cell system [14] produced only low and non-specific cell-associated radioactivity. Under the conditions of the assay, we should have been able to detect 5000 or more toxin-binding sites if the affinity constant was similar to the Vero cell system. Whereas our data do not rule out non-specific uptake, there are several suggestions that toxicity is receptor-mediated. For example, fragment A of DT at 100 μg/ml was not toxic to reticulocytes (data not shown). Since it is generally believed that fragment A does not interact with the DT receptor [1], fragment A-induced toxicity represents a measure of non-specific delivery processes. Therefore, our negative results with the fragment A are indicative of a receptor-mediated process with the whole toxin.

Further support for receptor-mediated toxicity is the pattern of protection with nucleotides. There is a specificity for this effect as regards the number of phosphates on the nucleotide and the type of base. Insofar as we could determine (table 2, and data not shown), protection of reticulocytes by nucleotides exhibits the same specificity seen in established receptor-related systems [10, 15, 16].

Another class of agents noted for their protective efficacy from DT are metabolic inhibitors [7–9]. Although it was originally speculated that these agents inhibited internalization of the toxin [7], more recent evidence points to a drug-induced loss of cell surface receptors as the mechanism of protection [9]. In any case, those metabolic inhibitors found to protect from DT in receptor-related systems also provided protection to reticulocytes. Similarly, metabolic inhibitors not effective in these other systems did not work with reticulocytes. As noted in the results, an exception to this pattern was dinitrophenol. This agent provided good protection with reticulocytes, but was ineffective in other systems [7–9]. Whereas we have no explanation for this observation, we consider it especially interesting, since it is generally accepted that dinitrophenol acts by inhibiting mitochondrial oxidative phosphorylation, yet reticulocytes have few or no mitochondria [17].

A very effective DT-protective agent in other cell systems is ammonium chloride [6–8, 11–13]. Whereas it was originally believed that ammonium chloride worked by maintaining DT on the cell surface [7], more recent studies have thrown serious doubt on that interpretation [12, 13]. In any event, while we could demonstrate protection, dose-response studies (fig. 2 and data not shown) indicated that ammonium chloride was much less effective in the reticulocyte system than in other cell lines. Furthermore, the apparent requirement for antibody after drug washout [7, 12, 13] was not observed with the reticulocytes (fig. 3). Although it is unclear how ammonium chloride protects other cell lines from DT, there are many studies documenting the lyso-

somotropic effects of the drug [18–20]. The results obtained with reticulocytes might well reflect apparent lack by the cell of that organelle with an accompanying insensitivity to lysosomotropic drugs. Further support for this explanation may be found in the lower DT-protective efficacy of other lysosomotropic amines such as methylamine (fig. 2) and chloroquine (data not shown).

Probably the most intriguing observation in this study was the insensitivity of reticulocytes to unnicked DT. It was reported earlier that HeLa cells were not sensitive to unnicked DT, although no data were presented [21]. We have been unable to confirm that report with the HeLa cell line or any other of several lines we use in this laboratory. Thus, we believe this is the first fully documented instance of a cell which responds differentially to nicked and unnicked DT.

There are two likely explanations for the insensitivity of reticulocytes to unnicked DT. First, it is possible that reticulocytes lack the enzyme which is responsible for nicking the toxin. In the maturation process, reticulocytes undergo the loss of virtually all organelles and it may be that certain specific proteins are also lost. The second possibility is that the nicking enzyme remains present, but that it is not located or packaged appropriately. Considering location, there is precedent for movement or repositioning of proteins in phenylhydrazine-induced rabbit reticulocytes [22]. From the packaging standpoint, it is known from electron microscopic examination that reticulocytes have few if any lysosomes [17, 23, J. L. Middlebrook & J. D. White, unpublished observations]. On the other hand, lysosomal enzyme activities have been demonstrated in reticulocytes [24]. In preliminary experiments performed here (J. L. Middlebrook & P. G. Canonico, unpublished observations), sucrose density-gradient analysis showed only a small fraction of the total lysosomal enzymatic activity of reticulocytes to be found at the density expected for that organelle. Thus, whereas reticulocytes apparently have lysosomal enzymes, it does not appear that they are packaged in a lysosome-like structure to which toxin could be delivered and processed.

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